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BIOSYNTHESIS OF ENZYMES BY SOLID SUBSTRATE FERMENTATION

I. Production of CM-Cellulase by Trichoderma viride

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Abstract. CM-cellulase formation by growing *T. viride* on wheat bran, maize bran, rice husk or bagasse was studied. Of all substrates, however, wheat bran proved to be the most suitable substrate for enzyme synthesis. Effect of various diluents was investigated, but enzyme production was maximum when CMC (0.5%) + mineral salt solution was used to dilute the wheat bran or other substrate in 1 l conical flasks. CM-cellulase production by the mould was induced by CMC ; addition of corn steep liquor showed an inhibitory effect on mould growth. Partial replacement of wheat bran by penicillin waste mycelium did not improve CM-cellulase formation. The addition of carbon sources such as glucose, soluble starch and maltose to wheat bran cultures were also investigated. Enzyme extraction remained about the same when tap water, phosphate buffer or sodium chloride solution was employed.

Solid substrate fermentation is defined as any fermentation in which the substrate is not liquid, the organisms grow on substrates such as rice, wheat, or wheat bran. This process, known as "Koji" is very common in Japan and it is used to produce various enzymes by growing moulds on cereals or on their brans. In the USA, however, this process is known as 'solid state fermentation'. This technique is actively being used for the study of mycotoxin formation by growing moulds on cereals or peanut. Merits and demerits of this newly developed technique have been reported by Hasseltine.[1]

Wheat bran or brans of other cereals are abundantly available in Pakistan. In the present work, therefore, attempts have been made to employ the technique of solid substrate fermentation using wheat bran, maize bran or rice husk in conical flask for the production of enzyme cellulase by *T. viride* on a laboratory scale. CM-cellulase and cellulase production by microorganisms is of great significance in view of the importance of this enzyme in deterioration of wood and textiles and in hydrolyzing cellulosic wastes to fermentable sugar which in turn can be used for single-cell protein. The enzymes also increase the digestibility and nutritive value of coconut and carrot by attacking the cell wall,[2] and find extensive applications as a digestive aid in pharmaceutical preparations. Moreover, the cellulase enzyme has also been incorporated in the preparations for quick digestion in sewage tanks, thus solving the pollution problems. Cellulase is an induced enzyme. The factors studied in the present investigation of CM-cellulase are selection of diluent, ratio of diluent to wheat bran, supplementation of carbon and nitrogen sources to the wheat bran substrates ; the conditions necessary for the extraction of enzyme from the wheat bran cultures have also been determined.

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Materials and Methods

Organisms. The strain of *Trichoderma viride* QM-6a, obtained from the U.S. Army, Natick Laboratories was used in the present study. The culture was maintained on potato-dextrose-agar medium incubating at 30 ± 2° for 3-4 days for maximum sporulation and then kept in the refrigerator.

Inoculum Preparation. The spores from 5-7 days old cultures were wetted by adding 10 ml of 0.005% manoxol O. T. (diacetyl ester of sodium sulphosuccinic acid) to each slant. The spores were scraped with a loop and the tubes gently shaken. The supernatent containing spores was decanted off aseptically and the suspension was used as an inoculum.

Fermentation Procedure. 20 g of wheat bran (or other substrate) was transferred to 1 l conical flask and diluted by adding 40 ml of diluent (unless otherwise mentioned), the composition of which is described below. The flasks, plugged with cotton wool, were sterilized in an autoclave at 121° for 15 min. The cooled bran was inoculated with 1 ml spore suspension and the wheat bran cultures were incubated at 30 ± 2° for 72 hr. The flasks were shaken twice daily. The wheat bran cultures in triplicate were run in parallel and the average results of CM-cellulase activity/g of wheat bran substrate were reported.

Diluents. Effect of the following solutions (as diluent for wheat bran) on the CM-cellulase synthesis was investigated. Generally 0.5% CMC + mineral salt was used to dilute the wheat substrate. 1. .0.5% CMC (sodium salt of carboxymethyl cellulose degree of substitution 0.45 ± 0.1, prepared in the Laboratory). 2. 0.5% CMC + Phosphate

buffer (K_2HOP_4 3.5 g/l and KH_2PO_4 1.5 g/l) pH 7.4. 3. 0.5% CMC + mineral salt solution consisting of $NaNO_3$ 3.00; KH_2PO_4 1.00; Tween 80 1 ml; $MgSO_4$ $7H_2O$ 0.50; KCl 0.50; $FeSO_4$. $7H_2O$ 0.01; Peptone 0.50 and CMC 5.00 g/l pH 7.0. 4. 0.5% CMC in water pH 7.0. 5. Distilled water pH 7.0.

Preparation of Enzyme Extract. The enzyme was extracted by adding 200 ml of distilled water to the wheat bran cultures in each flask. The flasks were shaken on a rotary shaker for 1 hr at 30 ± 2° room temperature. The mould bran suspension was filtered using Whatman filter paper and the filtrate was analysed for CM-cellulase activity.

Analytical Method. 1 ml of culture filtrate was incubated with 2.5 ml of 1% CMC, pH 4.6, at 37° for 1 hr. Glucose was determined by the dinitrosalicylic acid method.[3] One unit of cellulase was defined as that releasing 1.0 mg of glucose from 1% CMC in 1 hr at 37°, pH. 4.6.

Results

Fig. 1 shows the rate of enzyme synthesis by *T. viride* in wheat bran substrate moistened by adding 30 ml of 0.5% CMC + mineral salt solution. CM-cellulase formation reached maximum, *i.e.* 14.2 units/g. wheat bran, 72 hr after spore inoculation. Further increase in the incubation period resulted in the decrease of enzymic activity. In subsequent experiments, therefore, wheat bran cultures were incubated for 72 hr for enzyme formation.

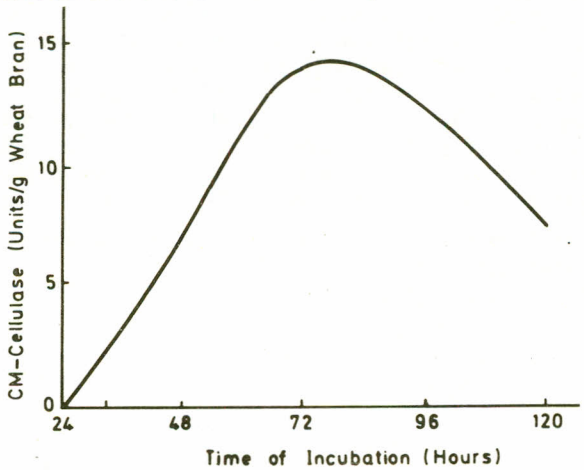

Fig. 1. Rate of CM-cellulase synthesis by *Trichoderma viride* QM-6a.

Selection of Wheat Bran Diluent Ratio. The moisture content in solid substrate fermentation is of great importance as excess of water affects the porosity of the wheat bran cultures ; thus fully aerobic conditions are not maintained. Wheat bran (20 g) contained in 1 l conical flasks was moistened by adding 0.5% CMC + mineral salt solution. The ratios of wheat bran to diluent were kept at 1 : 1; 1 : 1.5 ; 1 : 2; 1 : 2.5 by adding 20, 30, 40 and 50 ml of diluent to each flask respectively. (Table 1). CM-cellulase activity 72 hr after inoculation, was 14.15 and 14.93 units/g wheat bran when diluent ratios were 1 : 1.5 and 1 : 2. Further increase in the ratio of wheat bran to diluent.

TABLE 1. SELECTION OF WHEAT BRAN DILUENT RATIO FOR CM-CELLULASE SYNTHESIS BY *Trichoderma viride* QM-6a.

Diluent ml	Wheat bran : Diluent ratio	CM-cellulase units/g wheat bran
20	1 : 1	13.78 ± 0.24
30	1 : 1.5	14.15 ± 0.32
40	1 : 2	14.93 ± 0.27
50	1 : 2.5	12.55 ± 0.14

i.e. 1 : 2.5 resulted in the decrease of enzyme production, *i.e.* 12.55. Wheat bran-diluent ratio of 1 : 2 therefore, was used throughout the present work.

Extraction of CM-Cellulase from Wheat Bran Cultures. Extraction of CM-cellulase from wheat bran cultures was studied using phosphate buffer (pH 7.4). 0.9N sodium chloride (pH 5.4) solution or distilled water (pH 7.0) (Table 2). The wheat bran cultures were shaken for 1 hr after adding 200 ml of solution or water on a rotary shaker. The culture filtrates were analysed for enzymic activity. CM-cellulase extraction was better with water, *i.e.* 12.98 units/g. wheat bran than with phosphate buffer (11.93 units/g wheat bran) or sodium chloride solution. In subsequent experiments, therefore, distilled water was used for the extraction of CM-cellulase from wheat bran substrate.

Selection of Diluent. As medium composition greatly influences the enzyme formation, CM-cellulase production was investigated by moistening the wheat bran by various diluents (Table 3). The enzymic activity by simply adding water was 3.07 unit/g wheat bran and it was increased to 6.68 units/g wheat bran when CMC (0.5%) was used for moistening wheat bran. The enzyme formation, however, was 5.53 units/g when wheat bran was diluted with phosphate buffer containing CMC (0.5%) (pH 7.4). Thus CMC acted as inducer for enzyme synthesis by *T. viride*. The addition of mineral salts to CMC - water solution, however, greatly improved the production of CM-cellulase, *i.e.* 12.38 units/g. Thus adequate supply of minerals was essential for maximum cellulase formation by mould.

TABLE 2. STUDY OF DIFFERENT SOLUTIONS FOR EXTRACTION OF CM-CELLULASE FROM WHEAT BRAN CULTURES.

Solution for extraction	CM-cellulase units/g wheat bran
0.9N sodium chloride solution pH 5.4	10.45 ± 0.35
Phosphate buffer, pH 7.4	11.93 ± 0.85
Distilled water, pH 7.0	12.98 ± 0.32

TABLE 3. EFFECT OF VARIOUS DILUENTS ON CM-CELLULAS SYNTHESIS BY *T. viride* QM-6a.

Diluent solution	CM-cellulase units/g wheat bran
0.5% CMC-corn steep liquor	0.00 ± 0.00
0.5% CMC-phos. buffer, pH 7.4	5.53 ± 0.40
0.5% CMC-mineral salts solution	12.38 ± 0.57
0.5% CMC-water, pH 7.0	6.68 ± 0.76
Distilled water, pH 7.0	3.07 ± 0.45

CMC = carboxymethyl-cellulose.

Effect of Carbohydrates. Effect of adding carbohydrates such as glucose, maltose or soluble starch in CMC-mineral salt solution as diluent on the production of enzyme was also investigated (Table 4). The CM-cellulase synthesis was decreased by incorporating anyone carbohydrate in the diluent as compared with the control cultures. The enzyme activity in presence of glucose, soluble starch and maltose, was 9.91, 12.47 and 13.35 units/g wheat bran respectively. In control cultures, however, CM-cellulase production was 15.08 units/g wheat bran.

Penicillin waste mycelium, a by-product of penicillin factory at Daudkhail is a rich source of protein. Partial replacement of wheat bran by penicillin waste mycelium reduced the enzyme formation (Table 5). Rice husk was also evaluated for its use as substrate for CM-cellulase production by *T. viride*.

The ratio of rice husk to diluent was kept at 1:1.5 as the substrate become pasty with lower porosity by increasing the volume of diluent. The amount of rice husk was 20 g. Cellulase activity in rice husk was much lower (5.93 units/g), as compared with wheat bran (13.90 units/g) (Table 6).

TABLE 4. EFFECT OF CARBOHYDRATES ON C-M CELLULASE PRODUCTION BY *T. viride* QM-6a.

Diluent mineral salt solution + carbohydrate	CM-cellulase units/g wheat bran
1% Glucose	9.91 ± 0.39
1% Soluble starch	12.47 ± 0.59
1% Maltose	13.35 ± 0.58
Control	15.08 ± 0.24

TABTE 5. EFFECT OF PARTIAL REPLACEMENT OF WHEAT BRAN WITH PENICILLIN WASTE MYBELUM FOR CM-CELLULASE BIOSYNTHESIS BY *T. viride* QM-6a.

W.B. (g)	P.W.M. (g)	CM-cellulase units/g substrate
14	6	8.02 ± 0.40
16	4	10.40 ± 0.59
18	2	12.34 ± 0.60
20	0	13.95 ± 0.22
0	20 (Rice husk)	5.93 ± 0.78

Further studies were also made on CM-cellulase production by *T. viride* using maize bran, bagasse and spent wheat bran and also partially replacing them by wheat bran (Table 6). CM-cellulase production was lesser in maize bran (8.22 units/g) as compared with wheat bran alone (14.65 units/g) and it was increased with the increase in the concentration of wheat bran. Mould growth on bagasse substrate was slow and the cultures were incubated for 96 hr instead of 72 hr. The CM-cellulase activity was 8.10 units/g and the enzyme synthesis was improved with the increase of wheat

TABLE 6. EFFECT OF PARTIAL REPLACEMENT OF WHEAT BRAN BY MAIZE BRAN, BAGASSE AND SPENT WHEAT BRAN FOR CM-CELLULASE PRODUCTION BY *T. viride* QM-6a.

O.S. (g)	W.B. (g)	CM-cellulase units/g substrate			
		*M.B.	*S.W.B.	*B_I	†B_{II}
20	0	8.22 ±0.22	10.27 ±0.33	0.00 ±0.00	8.10 ±0.13
15	5	8.33 ±0.24	11.97 ±0.03	3.05 ±0.22	10.67 ±0.33
10	10	10.57 ±0.37	13.52 ±0.14	5.18 ±0.47	12.70 ±0.44
5	15	13.28 ±0.69	13.90 ±0.24	9.87 ±0.12	14.40 ±0.57
0	20	14.65 ±0.41	14.74 ±0.06	16.37 ±0.05	16.01 ±0.25

*Samples analysed after 72 hours incubation at 30 ± 2° C; W.B. = wheat bran; M. B. = maize bran; S.W.B. = spent wheat bran; O.S. = other substrate; B=bagasse. † Samples analysed after 96 hours incubation at 30 ± 2° C.

bran. Maximum activity of CM-cellulase however, was with wheat bran substrate alone. Attempts were also made to use spent wheat bran for the production of CM-cellulase. The amount of enzyme synthesized was in spent wheat bran substrate (10.27 units/g) as compared with wheat bran alone.

Discussion

The present work shows that wheat bran is more suitable substrate for mould growth and CM-cellulase synthesis by *Trichoderma viride* as compared with other substrates such as maize bran, rice husk or bagasse. Moreover, partial replacement of wheat bran, in solid substrate fermentation, by maize bran, rice husk, bagasse, spent wheat bran or penicillin waste mycelium reduced the enzyme formation. The superiority of wheat bran as a substrate for enzyme production is in agreement with the findings of other workers.[4,5] The important factor seems to be the chemical composition and porosity of the wheat bran. Enzyme production was maximum 72 hr after spore inoculation, further increase in the incubation period resulted in the decrease of CM-cellulase activity probably due to proteolytic enzymes synthesized by the fungus. The optimum ratio of wheat bran to diluent was 1:1.5 to 1:2. In most of the experiments, however, wheat bran-diluent ratio was kept at 1:2. The extraction of enzyme CM-cellulase from the wheat bran cultures was studied using saline solution, phosphate buffer and distilled water. The enzyme extraction was slightly better with distilled water, similar reports have also been made by other workers.[6,7] The addition of CMC in the diluent was essential since CM-cellulase formation was quite low when simple water was added to moisten the wheat bran. This is in agreement with the findings of other investigators,[8,9,10,11,12] that CM-cellulase was best synthesized when fungi were grown on cellulose and its derivatives which acted as enzyme inducers. The enzyme production was further increased (almost doubled,) when mineral salts were added in the diluents. Manzes *et al.*[13] have also reported the stimulatory effect of mineral salts in synthetic media for CM-cellulase production by *T. viride*. The incorporation of carbohydrates such as glucose, maltose and soluble starch in the diluents and their effect on enzyme synthesis was also investigated. No improvement in enzyme production was found by the addition of carbohydrates. Glucose addition reduced the CM-cellulase formation. Such reports of the inhibitory effect of sugar on CM-cellulase production have also been made by other workers.[8,14,15,16,17]

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